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LUNG TUMORS IN MICE RECEIVING DIFFERENT SCHEDULES OF URETHANE

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1. Introduction

Among the more common neoplastic reactions observed in the mouse is the primary pulmonary tumor. It was first described in 1896. (References to specific papers before 1955 are to be found in the review by Shimkin [12]). Autopsies performed by Wells, Slye and Holmes on 147,132 mice uncovered pulmonary tumors in 2,865, or two per cent, of which 104 had metastasized.

The mouse became a favorite experimental animal in cancer research through the efforts of geneticists, who developed many homozygous strains with a wide variety of neoplastic as well as other characteristics. No specific attempt was made to develop strains of high and low susceptibility to pulmonary tumors, but this phenotypic expression did become markedly segregated. Thus, there are strains such as A, in which almost all animals develop pulmonary tumors by 2 years of age, and strains such as C57 black, in which the occurrence of pulmonary tumors is a rarity. This rich material provided Heston with the opportunity of conducting his detailed studies on the relationship of genotypes to pulmonary tumors in mice. Recently, a single recessive major gene (*ptr*) conferring low susceptibility has been reported [3].

The induction of pulmonary tumors by exogenous carcinogens was first achieved by repeated applications of tar to the skin. The susceptibility to induced tumors was shown to parallel the spontaneous occurrence of the neoplasms; that is, induced tumors appeared most readily in strains that had the highest susceptibility to spontaneous pulmonary tumors.

Andervont in 1935 began his extensive studies on pulmonary tumors in mice, and showed that this reaction could be used as a test medium for a wide variety of chemical carcinogens. The number of tumor nodules observed on the lung

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surface could be used as a quantitative expression of carcinogenic potency, in a manner similar to the consideration of bacterial colonies on an agar plate.

The rapid induction of pulmonary tumors with carcinogenic polycyclic hydrocarbons facilitated studies directed toward histogenesis and the possible mode of action of the neoplastic transformation. It was established that the tumors arose from the cells lining the alveoli of the lung. Pulmonary tumors induced by a wide variety of chemical carcinogens were morphologically indistinguishable from spontaneous tumors, and were monotonously similar in all strains and individuals. On transplantation under the skin, some of the tumors retained the adenomatous appearance, whereas other acquired a sarcomatous appearance or a mixed morphology.

Studies on the route of injection of polycyclic hydrocarbons indicated that the carcinogen had to reach the lung in order to produce tumors. Thus, intravenous injections of dispersions were the most effective route, whereas if the carcinogens were introduced subcutaneously in media that retained them at the site of injection, few tumors in the lung appeared. With dispersions of different particle size, the number of the lung tumors could be related to the amount of carcinogen that became impinged in the lung. An ingenious experiment demonstrated that the neoplastic reaction was dependent upon factors in the lung, genetic or otherwise. Lung tissue from high and from low pulmonary tumor susceptible strains was transplanted subcutaneously into hybrids of these strains. The host then received an intravenous injection of carcinogen. The lung tissue from the susceptible strain gave rise to lung tumors, whereas the lung tissue from the resistant strain was rarely the site of such tumors.

Studies to elucidate factors that influenced the neoplastic reaction derived the following negative information: males and females developed approximately equal proportions and numbers of lung tumors, and the reaction was not influenced by exogenous sex or corticoid steroids. Foster nursing of high lung tumor strains by resistant strain females, and vice versa, had no influence on spontaneous or hydrocarbon induced lung tumors. Reticulo-endothelial blockade by trypan blue did not modify the induction of lung tumors. The production of inflammation of the lung with intravenous ore particles, or infection with influenza A or the gray lung virus did not influence the production of lung tumors. Acute fasting did not influence lung tumor induction, but chronic underfeeding that restricted body weight gain did inhibit the appearance of spontaneous lung tumors.

We know of no acceptable evidence that a viral agent is involved in pulmonary tumors in mice, but intensive studies have not been pursued.

Until the importance of the newborn phase of life became known, comparisons of younger and older mice usually involved animals of, say, 2 months of age with those 8 or 10 months older. In this range the induction of lung tumors was not influenced by age. Up to 6 weeks, however, a profound influence of age became clear. Newborn animals were much more susceptible to the induction of pulmonary tumors than the adults. This factor is related to the relative inability

of the newborn animals to eliminate polycyclic hydrocarbons [5] and urethane [4].

The discovery that urethane (ethyl carbamate), a simple water soluble chemical long employed as a veterinary anesthetic, produced pulmonary tumors in mice provided an experimental system with many desirable features.

Haddow [10] has written entertainingly about the polydynamic nature of urethane and the multifarious biological responses to which it gives rise. It suppresses cellular division of sea urchin eggs, is carcinogenic for not only the pulmonary tissue of mice but also for the skin and liver, and augments the occurrence of leukemia. Dustin [7] also has reviewed the pharmacologic studies on urethane.

In recent work on primary pulmonary tumors in mice urethane has become the favorite carcinogen. Among the contributions were attempts to quantitate the reaction and to modify it by various means in order to gain insight into the possible mechanism of action of the carcinogenic transformation. The quantitative aspects have led to the proposal of several mathematical models of carcinogenesis. These, in turn, became frameworks for further experimentation and analysis.

The main purpose of this report is to present some data on the effects of various schedules of urethane injections on pulmonary tumor production. It is regretfully true that, to a biologist, the results to date are more confusing than informative. In the anticipation that during this working conference some new interpretations and ideas might arise, however, we shall present the figures as they are. For this we shall use a historical approach, a consecutive description. First of all, an introduction is needed of some observations and concepts that guided the experimentation.

2. Background of experiments

When an adult strain A mouse is exposed to urethane, usually in a dose of 1 mg per gram of body weight, given intraperitoneally as a single injection of a ten per cent aqueous solution, the chemical is quickly transported throughout the body. The metabolic intermediates of urethane are not known, but it is known that practically all of it is eliminated from the body within 24 hours [1]. It is localized in the mitochondria of the lung cells. Whether urethane itself, or some metabolic complex is the carcinogenic stimulus also is not established [2].

The mice undergo a period of anesthesia, and recover without untoward effects. It is known that anesthesia is not involved in carcinogenesis, since many hypnotics such as pentobarbital and ether have no carcinogenic activity.

Within a matter of a few days, morphological effects become visible in the lung [13]. These are manifest by a generalized, nonspecific increase in cellularity, so that by 4 weeks the total number of cells is approximately doubled. This hypercellularity is manifested by a spatial disorientation of the cells of the alveolar walls of the lung. Experiments with tritiated thymidine indicate that the number of labeled cells is reduced during the first day and then steadily

rises by 10 days to threefold or more of the number in unexposed mouse lungs [9].

The generalized hypercellularity appears to recede after 4 weeks, and to be replaced by focal, ill defined areas that have been designated as hyperplastic foci. By 3 weeks there also begin to appear microscopic foci of unmistakable adenomatous tumor. The number of recognizable tumor foci increases rapidly up to 7 weeks, and from then on the number remains fairly constant. These observations are based on serial sections; tumors recognizable on the surface by the naked eye have to undergo further growth, so that additional tumors become evident for 20 weeks or more.

The tumors are discrete, of a typical appearance in the gross as well as under the microscope, and undergo a rapid growth phase for the first 4 to 7 weeks, after which the growth rate decelerates. This is probably due to the replication being limited to the periphery of the organoid tumor spheres. The tumors are randomly distributed in the lung; there is no predilection for side, lobe, or any given area of the alveolar tissue.

Neyman, in examining the published reports of Shimkin and Polissar, was impressed by the fact that a series of events took place following a single "pulse" stimulus, and postulated that the hyperplastic foci represented "first order mutants" from a proportion of which eventual tumors arose through at least one additional mutational process.

It seemed reasonable to anticipate that some substantiation for this hypothesis could be generated by exploring the effect of different schedules of administration of the carcinogen. Previous work had shown that the strain A lung tumor reaction could discriminate between twofold doses of carcinogens such as 3-methylcholanthrene and of urethane. However, most published experiments indicated that the mean number of lung tumors per mouse was related directly to the total dose, whether administered in one dose or whether the same dose was divided into up to 5 daily doses. However, perhaps spacing the doses over a longer period would accentuate the reaction by accelerating a mutational continuum involving several steps.

Three experiments were therefore undertaken to explore these possibilities. They were planned by Shimkin and Neyman and performed under a contract with Gubareff and his associates in St. Louis.

3. Description of experiments

3.1. *Experiment 1.* Strain A/J mice, males and females 2 to 3 months of age and weighing 18 to 31 grams, were randomized into two groups in August, 1963. One group received a single intraperitoneal dose of 1.0 mg urethane per gram body weight, dissolved in water to produce a ten per cent solution. The second group received urethane in 12 injections, given 3 times per week, for a total dose of 1 mg per gram body weight. Urethane solution was prepared freshly for each injection, and the dose adjusted to the weight of the animal at the time of injection.

This was a preliminary experiment, and only two to four mice were killed at 4 to 18 weeks following the single or the last dose of urethane.

The results are presented in table I. It is clear that more lung tumors were induced by a single dose than by 12 injections spaced over 4 weeks.

TABLE I

EXPERIMENT 1

Dose and Schedule	Weeks after Urethane	No. of Mice	Mean No. of Lung Tumors per Mouse	No. of Lung Tumors in Individual Mice
1 mg per gm single dose	4	2	2.0	2, 2
	8	3	8.3	5, 7, 13
	12	4	8.8	5, 8, 10, 12
	14	4	10.8	5, 10, 14, 14
	18	4	18.3	12, 16, 21, 24
1 mg per gram in 12 doses (3 per week)	4	2	0.5	0, 1
	8	4	0.3	0(3), 1
	12	4	1.5	1, 1, 2, 2
	14	4	3.3	0, 3, 5, 5
	18	4	3.8	1, 3, 4, 7

3.2. *Experiment 2.* Strain A/J mice, males and females, 4 to 6 weeks old and weighing between 12 and 16 grams, were assigned by randomization to seven groups. They were given intraperitoneal urethane, prepared freshly for each injection, as indicated below, and all were killed at 22 weeks following the first injection.

Group 1: 1.0 mg per gram in a single injection.

Group 2: 1.0 mg per gram in 2 doses of 0.5 mg on Tuesday and Monday (6 day interval).

Group 3: 1.0 mg per gram divided into 12 doses, given on Monday, Wednesday and Friday for 4 weeks.

Group 4: 0.5 mg per gram given a single injection.

Group 5: 0.5 mg in 2 doses of 0.25 mg on Tuesday and Monday (6 day interval).

Group 6: 0.25 mg per gram given in a single injection.

Group 7: 0.25 mg divided into 4 doses, given on Tuesday, Thursday, Saturday and Monday (3 intervals of 2 days).

The results are presented in table II. They are difficult to reconcile with the findings of experiment 1. The decreased number of lung tumors when 1.0 mg is divided into 12 doses is no longer clear, although numerically there were fewer tumors as compared with the single dose schedule.

It is worthy of note that the relationships of dose to effect, when given in single doses, was almost embarrassingly excellent: 16.4 for 1.0 mg; 9.1 for 0.5 mg; and 4.4 for 0.25 mg. With the same doses divided into 2 injections 6 days apart,

the number of lung tumors was increased, from 16.4 to 23.4 for 1.0 mg and from 9.1 to 18.6 for 0.5 mg. A similar effect was seen in mice receiving 0.25 mg in 4 doses every other day, an increase from 4.4 to 11.8. For the two lower doses, therefore, the effects were *doubled* by dividing the dose. Adjustment for the time at sacrifice would accentuate the differences, which are statistically highly significant for the two lower doses without further adjustment.

TABLE II

EXPERIMENT 2

Dose and Schedule	No. of Mice	Mean No. of Lung Tumors per Mouse	No. of Lung Tumors in Individual Mice
1 mg once	34	16.4	7, 9, 9, 10, 10, 11, 11, 12(5), 13, 13, 16, 16, 17, 18(3), 19(5), 20, 21, 21, 22, 22, 23, 23, 24, 28
1 mg in 2 doses 6 days apart	33	23.4	7, 7, 12, 12, 15, 17(3), 19, 19, 20(4), 21, 22, 22, 24, 24, 25, 26, 26, 27, 27, 28, 28, 29, 34, 34, 36, 36, 37, 42
1 mg in 12 doses 3 times per week	34	14.3	7, 7, 8, 8, 9, 9, 10(3), 11, 11, 12, 13, 14, 15(6), 16(4), 17, 18, 18, 19(3), 20, 20, 22, 23
0.5 mg once	39	9.1	3, 4, 5(3), 6(4), 7(6), 8(4), 9(5), 10(3), 11(4), 12, 13, 13, 14, 14, 15, 16, 19
0.5 mg in 2 doses 6 days apart	39	18.6	10, 11, 14, 14, 15, 16(6), 17(3), 18(6), 19(6), 20, 21(3), 22(4), 23(3), 25, 26
0.25 mg once	39	4.4	1, 1, 2(5), 3(5), 4(8), 5(8), 6(7), 7(3), 8
0.25 in 4 doses 2 days apart	39	11.8	5, 6, 7(4), 8(7), 9, 9, 10, 10, 11, 11, 12(5), 14(6), 16(3), 17, 18, 18, 19, 19, 20

The results of the different effects of the 12 divided doses and the 2 or 4 divided doses appeared to defy any single interpretation. Two possible explanations had to be invoked, one of more rapid elimination of the carcinogen given in 12 doses, through the increased age or induced enzyme mechanism, and the other of a reinforcement of the carcinogenic continuum by a small number of repeated doses.

3.3. *Experiment 3.* Experiment 1 was therefore repeated and extended. Strain A/J mice, males and females 4 to 6 weeks old and weighing 11 to 16 grams, were randomized into two groups.

One group received a single intraperitoneal dose of 1.0 mg urethane per gram body weight. The second group received 12 injections, 3 times per week, for a total dose of 1.0 mg per gram. The animals were sacrificed at 20, 28 and 34 weeks following the single or the last dose of urethane, and the number of lung tumors determined.

The results are presented in table III. It is clear again that more lung tumors were induced by the single dose than by the 12 injections spaced over 4 weeks. The differences in each instance, adjusting for the 4 week longer exposure to the total dose in the single injection group, are statistically significant.

TABLE III

EXPERIMENT 3

Dose and Schedule	Weeks after Urethane	No. of Mice	Mean No. of Lung Tumors per Mouse	No. of Lung Tumors in Individual Mice
1 mg per gm. once	20	34	11.5	2, 3, 4, 5, 6(3), 7, 8(3), 10, 10, 11(4), 12(3), 13(4), 14, 14, 15, 16, 18(4), 20, 24
	28	29	17.7	1, 11, 12(4), 13, 15, 15, 16(6), 17, 17, 18, 18, 20, 21, 22, 23, 23, 24, 24, 26, 30, 31
	34	27	23.7	10, 15, 16(3), 17, 17, 19, 20, 21, 21, 23(3), 24(3), 25(3), 29, 29, 30, 32, 35, 37, 46
1 mg in 12 doses 3 per week	20	30	7.0	2, 2, 3, 3, 4(4), 5(3), 6(4), 7(5), 8, 8, 12(6), 15, 17
	28	31	9.5	3, 4, 4, 5, 6, 7(6), 8, 9(4), 10(3), 11(5), 12, 12, 13, 15(3), 19
	34	30	13.6	3, 5, 7, 8(3), 9, 9, 10, 11, 11, 12(4), 13, 13, 14, 14, 15, 16, 17, 17, 18, 18, 19, 19, 20, 24, 24

At the time this experiment was performed, we were not aware of the fact of slower excretion of urethane by very young animals. In retrospect, one interpretation is that the longer spacing of urethane over 4 weeks was given to nonadult animals which excreted the carcinogen at a progressively greater rate. In effect, therefore, the 12 dose group received less urethane than was given in a single dose when the animals were less capable of excreting the compound. Another possible interpretation is that the animals on repeated doses "learned" to eliminate the carcinogen more rapidly through some process akin to an induction of an enzyme.

3.4. *Experiment 4.* An attempt was made to repeat the observations of the three previous experiments and to fill out some of the cells in the already complicated matrix of doses and schedules. This experiment, as well as experiment 5, was performed at Fels Research Institute by Mr. Ronald Wieder and Mrs. Dianne Marzi.

Strain A mice of both sexes 2 to 3 months old and weighing 23 to 25 grams random allocation to eight groups. All mice were sacrificed at 131 days after the first injection.

Group 1: 1.0 mg per gram body weight in 1 injection.
Group 2: 1.0 mg in 10 doses, given 3 times per week.
Group 3: 1.0 mg in 10 daily doses.
Group 4: 0.5 mg in 1 injection.
Group 5: 0.5 mg in 2 doses 1 week apart.
Group 6: 0.25 mg in 1 injection.
Group 7: 0.25 mg in 10 doses given 3 times per week.
Group 8: 0.25 mg in 10 daily doses.

The results are given in table IV. Despite the small groups, the relationship to doses given in single injections remains excellent. There does appear a significant depression in the mean tumor yield when 1.0 mg is divided into 10 thrice weekly or daily doses, as in experiments 1 and 3. There is also a suggestion of such effect when 0.25 mg was divided into 10 thrice weekly or daily doses, although obviously the differences are not significant statistically. But the lack of confirmation of the enhancement of effect with the 0.5 mg dose divided into 2 doses one week apart, seen in experiment 2, is now a disappointment.

TABLE IV

EXPERIMENT 4

Dose and Schedule	No. of Mice	Mean No. of Lung Tumors	Mean No. of Small Tumors	No. Lung Tumors in Individual Mice
1.0 mg once	12	20.2	19.0	9, 16, 19, 19, 20, 20, 21, 21, 23(3), 27
1 mg in 10 doses 3 per week	9	11.3	10.3	6, 8, 10(3), 12, 14, 15, 16
1 mg in 10 daily doses	9	15.9	14.7	8, 9, 10, 11, 17, 21, 22, 22, 23
0.5 mg once	13	7.5	6.7	3, 5(4), 6, 7, 8, 8, 10, 11, 11, 14
0.5 mg in 2 weekly doses	12	7.0	7.6	3, 4, 4, 6, 7(3), 8, 9, 10, 12, 18
0.25 mg once	10	5.3	4.7	2, 3, 3, 4, 5, 5, 6, 7, 7, 11
0.25 mg in 10 doses (3 per week)	12	4.9	4.4	3(3), 4(4), 5, 7(3), 8
0.25 mg in 10 daily doses	11	4.0	3.4	1, 2, 3, 3, 4(4), 5, 5, 9

In the analysis of this experiment, separate consideration was made of lung nodules up to 1 mm in diameter. The results remained identical, with the mean values of the number of lung tumors being reduced by approximately 1 for the 1.0 mg groups, and by 0.6 for the 0.25 mg groups.

What went wrong, or what the explanation of these divergent observations might be, is a puzzle to a biologist.

The possibility of enhancing the carcinogenic effect by properly spaced repeated doses of urethane again becomes attractive upon reviewing an experiment reported by Henshaw and Meyer in 1945 [11]. They gave one group of 20 strain A mice 4 weekly "anesthetic" doses of urethane. Upon sacrifice 25 weeks later, these mice had an average of 25.7 lung tumors per animal; however, when the tumors were classified by size, the animals had an average of 18.5 large tumors and 7.3 small tumors. The second group of 20 mice received 2 weekly injections and then 2 more weekly injections after a 7 week rest period. On sacrifice 25 weeks following, they had an average of 35.1 tumors, but these included an average of 18.5 large tumors and 16.6 small tumors per mouse. The difference in the number of *small* tumors in these two groups is highly significant statistically, although the total number difference does not meet statistical significance.

The effect reported by Henshaw and Meyer suggested that the selection of a sufficiently long interval between injections, of 7 weeks, accelerated an incomplete neoplastic reaction induced by the first series of injections of urethane. An attempt to repeat their observations seemed in order.

3.5. *Experiment 5.* Swiss mice were used because the supply of strain A mice was inadequate. The animals were randomized into nine groups, with further identification of males and females, and by two weight ranges: 16 to 18 grams and 23 to 25 grams. The lighter animals were 6 to 8 weeks old, and the heavier ones, about 12 weeks old.

The purpose was to explore the effect of the interval between two exposures to urethane, according to the experiment of Henshaw and Meyer. Thus, four groups received 0.5 mg per gram body weight on the same day, May 12, 1965. These groups contained equal numbers, 6 each, of males and of females, and of the younger and of the older animals, so that each group initially had 48 mice. The second injection of 0.5 mg per gram, as determined by weight at the time, was given after intervals of 1, 2, 4 and 7 weeks. Initially and at each of the second injection dates an equal group of animals distributed in the same manner by sex and age, received single injections of 1.0 mg per gram. As previously, freshly dissolved urethane in water to yield a 0.1 per cent solution was employed.

All animals were sacrificed at 20 weeks following the only or the second injection of urethane.

To recapitulate, the nine groups consisted of the following:

Group 1: 1.0 mg on 5/12; killed 9/29;
Group 2: 0.5 mg on 5/12 and 5/19; killed 9/29;
Group 3: 1.0 mg on 5/19; killed 10/6;
Group 4: 0.5 mg on 5/12 and 5/26; killed 10/13;
Group 5: 1.0 mg on 5/26; killed 10/13;
Group 6: 0.5 mg on 5/12 and 6/19; killed 10/27;
Group 7: 1.0 mg on 6/19; killed 10/27;
Group 8: 0.5 mg on 5/12 and 6/30; killed 11/17;
Group 9: 1.0 mg on 6/30; killed 11/17.

The results are presented in table V. Analysis by sex and by the two age groups revealed no consistant or significant differences, so that the data were combined in these regards. On a total basis, 203 males had 3269 tumors, for a mean of 16.1 tumors per animal; 200 females had 3924 tumors, or 19.6 per animal. The younger mice had 3243 tumors in 201 animals, or 16.2 per animal, and the 202 older ones had 3950 tumors, for a mean of 19.5. These differences are in reverse of what was anticipated.

Analysis of the tumors by size also did not influence the results. Thus, consideration of tumors up to 1 mm in diameter yielded the same conclusions as the total tumor count.

There does seem to be an increased number of tumors when the dose was divided, one week apart and 7 weeks apart. The difference between single and

TABLE V

EXPERIMENT 5

Dose and Schedule	No. of Mice	Mean No. of Lung Tumors per Mouse	Mean No. of Small Tumors per Mouse	No. of Lung Tumors in Individual Mice
1 mg once	44	17.4	13.7	4, 5, 6, 7, 7, 8, 9(3), 10(5), 11, 11, 12, 13, 13, 14, 15, 15, 16(5), 17, 18, 18, 19, 20, 21, 22, 25, 27(3), 28, 34, 47, 47, 51
0.5 in 2 doses 7 days apart	47	21.3	17.9	2, 3, 4, 5, 5, 6, 7(3), 10(3), 11, 12, 13, 14, 14, 15(5), 16(3), 17, 18, 18, 19, 20, 22, 23, 24(3), 27, 29, 31, 32, 34, 34, 35, 37, 38, 41, 49, 54, 59
1 mg once	45	15.0	12.3	2, 2, 3, 4, 5, 7(4), 8, 9(5), 10, 10, 11(3), 12(4), 13, 13, 14, 14, 15, 15, 17, 18, 19, 22, 22, 24, 24, 26, 27, 30, 30, 31, 35, 52
0.5 in 2 doses 14 days apart	44	15.9	13.4	2, 3, 3, 4, 5, 8, 9(6), 10, 10, 11(3), 12, 12, 13, 13, 14(3), 15(3), 17, 17, 18(3), 19, 20, 20, 21, 25, 25, 27, 28, 31, 33, 46, 51
1 mg once	45	20.1	17.0	4(3), 5, 6, 7, 7, 8, 8, 10(3), 11(4), 12, 12, 13(3), 14, 15, 15, 16, 17, 18, 19, 20, 21, 26, 28, 28, 30, 31, 31, 32, 35, 37, 40, 43, 44, 45, 50, 56
0.5 in 2 doses 28 days apart	42	18.8	15.7	6, 7, 7, 8(4), 9(4), 10, 10, 11, 11, 12, 13, 14, 14, 17(4), 18, 18, 19(3), 20, 20, 22, 25, 26, 27, 28, 32, 33, 33, 41, 43, 45, 53
1 mg once	47	17.9	15.0	2, 4, 5, 6, 7(3), 9, 9, 10(4), 11(6), 12, 12, 13, 13, 14(3), 16(4), 17, 18, 19, 21, 22, 23, 24, 24, 27, 28, 38, 38, 39, 41, 43, 51, 52
0.5 mg in 2 doses 49 days apart	43	21.2	17.3	2, 4, 5, 7, 9(3), 10, 11(3), 13, 13, 14(3), 15, 16, 16, 17, 17, 18, 18, 19, 20, 20, 21, 24, 25, 25, 29, 30, 31, 31, 32, 32, 33, 34, 36, 36, 37, 50, 72
1 mg once after 49 days	47	14.4	13.4	1, 2, 5(3), 6, 6, 7(4), 8, 8, 9(4), 10, 10, 11(3), 12, 12, 13, 13, 14(3), 15, 15, 16, 16, 18, 18, 19, 19, 20, 20, 22, 23, 25, 27, 28, 33, 34, 58

the 7 week interval group is statistically significant. However, the differences are certainly not striking, being in the order of 15 to 20 per cent higher in the two injection groups than in the single injection groups. The difference is not evident and, in fact perhaps slightly reversed, when the interval was 14 days. Of course, this is also referable to the high mean count at that point in the single injection group.

There is no reason, of course, why a constant linear increase in lung tumors should occur with the two injection schedule throughout the course of the reaction. It may be found eventually that the biochemical events in the lung tissue indeed go through several phases or undulations, with peaks at 1 and at 7 weeks.

It should be noted, in passing, that the Swiss mice were as susceptible to lung

tumor induction as the strain A mice. The wider variability and deviations from the mean indicate that the Swiss mice are less homogenous than the A mice.

4. Discussion

It is sad to admit that the question of the effect on lung tumor production by various dose schedules of carcinogens is not clearly answered by the experiments reported here, or by data that exist in the literature. There are, instead, a series of hints or nonreproduced results that yield several possibilities. As in most biological investigations, there are probably more unknown variables than the defined ones, with the additional complication that the animal changes during the course of the investigation.

We invite the cogitation of our mathematical colleagues to suggest hypothetical interpretations that can be tested by further investigations. We feel, however, that better insight is probably to be derived from studies of the cellular population of the mouse lung rather than of the end result of the tumor nodule.

The lung of an adult mouse is estimated to be composed of some 500 million cells of several distinct types, of which the cells lining the alveoli represent but a small proportion. Even if this is estimated as one per cent of the total, this still represents 5 million cells that give rise to, say, up to 50 tumor nodules. An event that occurs once in 100,000 deserves being classed as being relatively rare. How can we identify this event, when it is obscured or diluted by 99,999 events that may be irrelevant or immaterial? This species of difficulty is exactly the same as is encountered by a biochemist, who seeks to identify some biochemical event, a biochemical neoplastic "lesion," on a cellular level, in a tissue homogenate.

Table VI outlines a few variables that have been studied in relation to the induction of primary pulmonary tumors in mice.

Among the newer studies that yield information on factors that modify the reaction are those of Foley and Cole [8], who showed that X-radiation inhibits the induction of lung tumors with urethane, and Duhig's observations [6] that thymectomy has no influence on the reaction elicited with Nitrogen Mustard. In this connection, Shimkin *et al.* [14] have examined the dose response relationships of the induction of lung tumors by a series of alkylating agents. This is a rather hardy tumor, not influenced by steroid hormones, nutrition and many other factors that affect other neoplastic reactions, and thus make them more attractive materials in cancer research because of these convenient investigational handles. Regretfully, in this list, the effects of schedules of doses must still be represented by a question mark.

Now: where do we go from here?

As indicated before, biologists are willing to set up more mice and to count and to measure more pearly white nodules that appear on the surface, if this is called for by statistical or biological conceptualization.

But, since we are now in the era of cellular and molecular biology, perhaps a

TABLE VI

FACTORS INFLUENCING INDUCTION OF LUNG TUMORS IN MICE

Tumors induced by polycyclic hydrocarbons H, urethane U, alkylating agents A or spontaneous S

Factors	Influence	Material
Heredity		
Strain	+++	S, H, U, A
Sex	0	S, H, U, A
New born state	++	H, U
Age	+	S, H, U
Foster nursing	0	S, H
Exogenous stimulus		
Chemical structure	+++	H, U, A
Vehicle	++	H, A
Route	++	H, A
Schedule	?	H, U
Nutritional		
Acute fasting	0	U
Chronic undernutrition	+	S
Immunologic		
RE blockade	0	H
Thymectomy	0	A
Inflammatory		
IV ores	0	H
Influenza A	0	S
Gray lung virus	0	U
Others		
X-ray	+	U
Colchicine	0	U

better approach would be to delve into the cellular levels of the reactions. This we intend to do, with the help of Dr. Baserga and other talents at our laboratories.

The first and most obvious investigation is to determine, in greater detail than suggested by Foley, Cole, Ingram, and Crocker [9], the cellular replication rates in mouse lungs exposed to urethane. Tritiated thymidine labeled cells need examination from two standpoints: what cells are involved, and what is the distribution of such labeling? Is it at random, or can a focal pattern involving the alveolar lining cells be deduced?

Tritiated thymidine is only the first step, and additional information would be forthcoming by double contrast radioautography.

One of the important peripheral yields of the cancer chemotherapy program has been the discovery of many antimetabolic chemicals that block specific pathways in the synthesis of nucleotides and proteins. Of particular interest in the lung tumor problem are Actinomycin D, which blocks messenger RNA, puromycin, which blocks protein synthesis, and cytosine arabinoside, which blocks DNA synthesis. What would be the effect of these agents on lung tumor production in mice? We propose to see.

A final comment about mathematical expressions of the carcinogenic process.

It would seem, to a biologist, that this is a complex continuum involving many steps. These steps do not cease at the conversion of the alveolar lining cells to an organoid mass of adenomatous cells. Morphologic observations certainly indicate that eventually some of these well organized structures become more aggressive, invade more actively, and finally acquire characteristics that empower the cells with the ability to metastasize.

But these further steps, so essential for our understanding of the neoplastic processes, are for future investigations.

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